American Journal of Pathology, Vol. 161, No. 6, December 2002

Serotonin Mechanisms in Heart Valve Disease II

The 5-HT$_2$ Receptor and Its Signaling Pathway in Aortic Valve Interstitial Cells

Jie Xu,* Bo Jian,* Richard Chu,† Zhibin Lu,* Quanyi Li,* John Dunlop,‡ Sharon Rosenzweig-Lipson,‡ Paul McGonigle,‡ Robert J. Levy,* and Bruce Liang†

From the Cardiology Research Laboratory, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania; the Departments of Medicine and Pharmacology,† University of Pennsylvania Medical Center, Philadelphia, Pennsylvania; and Wyeth-Ayerst Research,‡ Princeton, New Jersey*

Serotonin [5-hydroxytryptamine (5-HT)]-mediated cardiac valvular disease has been commonly observed in patients with carcinoid tumors. Previous research by others using reverse transcriptase-polymerase chain reaction demonstrated that aortic valve cells expressed predominantly 5-HT$_{2A/2B}$ receptors (5-HT$_{2A}$R). Related investigations by our group using sheep aortic valve interstitial cell (SAVIC) cultures demonstrated that 5-HT both up-regulates transforming growth factor (TGF)-β1 expression and activity, and also results in increased phospholipase C (PLC) activity. Thus, the present study investigated the hypothesis that the 5-HT signaling pathway in SAVICs involves 5-HT$_2$Rs with associated G-protein signal transduction. The objectives were to functionally characterize in SAVIC cultures the native serotonin receptor subtypes using specific agonists and antagonists, and to delineate the serotonin-signaling pathway. 5-HT administration caused a marked stimulation of PLC activity. SAVIC studies of specific agents that target the 5-HT$_2$R subtypes indicate that this response seemed to be mediated predominantly by 5-HT$_{2A}$Rs. Furthermore, the sheep 5-HT$_{2A}$R was identified by reverse transcriptase-polymerase chain reaction with sequence confirmation including comparisons to pig and human 5-HT$_{2A}$R. Extracellular signal-regulated kinase (Erk 1/2) is a signaling molecule downstream from the 5-HT$_{2A}$R. Both a protein kinase C inhibitor, GF109203X, and a Src inhibitor, PP1, attenuated 5-HT-stimulated Erk 1/2 activation. However, a 5-HT$_{2A}$R antagonist, MDL 100907, inhibited 5-HT up-regulation of PLC and TGF-β1, while having far less pronounced effects on Erk 1/2. In conclusion, these studies of the signal transduction activity of SAVICs in response to 5-HT have demonstrated that the 5-HT$_{2A}$Rs are the most functionally active of the 5-HT$_2$Rs in this cell type. Furthermore, 5-HT$_{2A}$Rs are also involved in 5-HT up-regulation of active TGF-β. 5-HT also mediated strong Erk 1/2 signaling via the MAP-kinase pathway, which was only in part because of 5-HT$_{2A}$R activity. Thus, major 5-HT Erk 1/2 signaling beyond that controlled by 5-HT$_2$Rs must involve other serotonin receptor types and/or secondary signaling events. *(Am J Pathol 2002, 161:2209–2218)*

Heart valve fibroplasia has been commonly seen in association with carcinoid tumors because of 5-HT secretion, or in association with ergotamine-induced valve disease.[1–3] Ultrastructural studies demonstrated that carcinoid-associated fibroplasia, which grossly consists of plaque-like endocardial thickenings, is composed of fibroblasts or myofibroblasts and a fibrous stroma, enriched in collagen, acid mucopolysaccharides, and microfibrils, but devoid of elastic fibers.[1,4] Previous experimental studies have also shown that serotonin can stimulate collagen synthesis in human valve interstitial cells.[5] Studies by our group have demonstrated that 5-HT up-regulates transforming growth factor (TGF)-β1 in sheep aortic valve interstitial cells (SAVICs) in culture;[6] these investigations have also provided indirect evidence that this up-regulation may occur through G-protein signal transduction. However, the mechanism of 5-HT-mediated cardiac valve disease is incompletely understood.

5-HT receptor expression in heart valves or their interstitial cells has been the subject of several recent investigations.[7,8] A total of 15 serotonin receptor subtypes have been discovered to date, and they may be subdivided further into seven subfamilies. All serotonin receptors, except for 5-HT$_3$, belong to the superfamily of G-protein-coupled receptors. Previous studies have reported that 5-HT$_{1B/1C}$ and 5-HT$_{2A/2B}$R subtypes are

Supported in part by grants from the National Institutes of Health (RO1 HL38118 to R. J. L., RO1 HL48225 to B. L., T32 HL07915 to R. J. L., J. X., and B. J.), the Wyeth-Ayerst Research (research grant to B. L.), and the William J. Rashkind Endowment of the Children's Hospital of Philadelphia.

Accepted for publication August 15, 2002.

Address reprint requests to Bruce Liang, M.D., Neag Distinguished Professor and Chief of Cardiology, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030. E-mail: bliang@uchc.edu.

expressed in aortic valve cells from human, porcine, as well as canine aortic valves.[7,8] Thus, in view of the well-established association of 5-HT with carcinoid cardiac valvulopathy, and the possibility of involvement of established receptor signaling pathways, we sought to carefully characterize 5-HT signaling in aortic valve interstitial cells (AVICs) with a long-term view toward both anticipating potential problems with serotonergic agents and the possibility of innovative therapies for heart valve disease. In the present studies, we investigated the hypothesis that our previous observation of 5-HT up-regulation of TGF-β1 in SAVICs is because of 5-HT_2R activity with associated G-protein signal transduction. Thus, we sought to functionally identify and characterize the 5-HT_2R subtype(s) in SAVICs. 5-HT_2R-mediated signaling pathways have been extensively studied in many cell types, such as mesangial cells, fibroblasts, and neurons.[9–11] However, no studies of this nature have been performed with valvular interstitial cells. Therefore, our study also sought to characterize the signaling pathway for the SAVIC 5-HT_2R.

Materials and Methods

Materials

Cell culture media consisting of M199 and Dulbecco's modified Eagle's medium supplemented with penicillin, streptomycin, and fetal calf serum were all from Life Technologies, Inc. (Rockville, MD). Serotonin was obtained from Sigma (St. Louis, MO). Phospholipase C (PLC) inhibitor (U73122), protein kinase C (PKC) inhibitor (GF 109203X), Src-family tyrosine kinase inhibitor (PP1), and MEK inhibitors (PD98059 and U0126) were from BioMol (Plymouth Meeting, PA). Western analysis apparatus, precast gels, and polyvinylidene difluoride membranes were from BioRad (Hercules, CA). Phospho-specific-p44/42 mitogen-activated protein kinase E-10 monoclonal antibody was purchased from New England Biolabs (Beverly, MA) and nonphospho-specific-p44/42 mitogen-activated protein kinase polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Selective 5-HT_2R antagonists/agonist (antagonists, MDL 100907, SB 242084, SB 206553, and the agonist, BW 723C86) were provided by Wyeth-Ayerst (Princeton, NJ). A replication defective adenovirus construct for overexpression of Gαq under the control of the cytomegalovirus promoter (AdCMV-Gαq) was provided by Dr. Morris Birnbaum (University of Pennsylvania School of Medicine, Philadelphia, PA), and was used as previously described by our group;[6] a green fluorescent protein (GFP) adenovirus, AdCMV-GFP, was used as a control.[6] Dr. Quanyi Li (Children's Hospital of Philadelphia) provided cDNA obtained from AVICs cultivated from a human aortic valve specimen that was removed from a 2-year-old male heart transplant patient with congenital aortic valve stenosis and a ventricular septal defect.

Isolation and Cultivation of SAVICs

Sheep aortic valves were obtained from female sheep, age 6 months to 2 years at the time of sacrifice as approved by Institutional Committee for the Use and Care of Animals (IACUC) of Children's Hospital of Philadelphia. Aortic valve leaflets were dissected and the endothelial cell layer was removed. The remaining tissues were cut by gentle rolling of the scalpel blade and were collected in a 15-ml Falcon tube containing 0.1% type I collagenase in M199 for incubation at 37°C for at least an hour with occasional vortex. Cells were then washed once and placed into culture plates in M199 supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged using 0.25% trypsin in 1 mmol/L ethylenediaminetetraacetic acid (Invitrogen, Carlsbad, CA) and used from passages 3 to 9.

PLC Assay

PLC activity was determined by a modification of the method from Berridge and colleagues.[12] Briefly, cultured SAVICs in 6-well plates (3.5×10^5 to 4.5×10^5 cells per well) were labeled with *myo*-[3H] inositol (2.5 μCi/ml) for 24 hours in inositol-free Dulbecco's modified Eagle's medium supplemented with 0.5% fetal bovine serum. After washing, cells were incubated in Dulbecco's modified Eagle's medium (with 15 mmol/L LiCl) with or without various agonists or antagonists or combinations of both for 30 minutes to 1 hour unless otherwise indicated. Cell membranes (inositol-enriched region) were extracted using chloroform/methanol/HCl (1:2:0.05, by volume). Inositol 1-phosphate (InsP1), inositol 1,4-bisphosphate [Ins(1,4)P2], and inositol 1,4,5-triphosphate [Ins(1,4,5)P3] were eluted sequentially into scintillation vials with 0.2 mol/L ammonium formate/0.1 mol/L formic acid, 0.6 mol/L ammonium formate/0.1 mol/L formic acid, and 1.0 mol/L ammonium formate/0.1 mol/L formic acid, respectively, on an anion-exchange column (AG1X8 resin, formate form). Radioactivity was determined on a Beckman scintillation counter (LS-3801; Beckman, Irvine, CA). Triplicate wells were used in every applicable control or treatment. At least three independent experiments were performed for each treatment.

A luciferase assay for TGF-β activity was performed using mink lung epithelial cells provided as a gift by Dr. D.B. Rifkin (New York University), that were stably transfected with a portion of the plasminogen activator inhibitor 1 promoter.[13] Equal numbers of quiescent SAVICs ($\sim 4 \times 10^5$ cells) cultivated on collagen-coated six-well plates were treated with 10 μmol/L of 5-HT for 24 to 72 hours. After incubation, medium (test sample) was collected, centrifuged, and assayed for TGF-β1 activity by PAI/L assay, as described by Abe and colleagues.[14] Mink lung epithelial cells were cultivated in the presence of test samples for 14 hours at 37°C to assay luciferase activity for active, and separately, total TGF-β activity (after heating at 80°C for 5 minutes).[13]

RNA Isolation

Confluent interstitial cells were trypsinized and homogenized. Total cellular RNA was extracted using a TRIzol extraction kit (Life Technologies, Inc.), according to the manufacturer's instructions. The RNA concentration was quantitated by absorbance at 260 nm. The integrity was assessed by electrophoresis of 1 μg on an ethidium bromide-stained, formaldehyde-agarose minigel.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Sequence

Using ~1 μg of total RNA, reverse transcription was performed with primer oligo-dT and reverse transcriptase (SuperScript First-Strand Synthesis System for RT-PCR kit, Life Technologies, Inc.). Because the full-length cDNA sequence of sheep serotonin receptor is not known, receptor sequences from several other species (human, mouse, and porcine) were compared and a consensus sequence was chosen for PCR primer design. Forward and reverse primers were designed for 5-HT$_{1A}$ (GACGGTCAAAAAGGTGGAGA, GCAGAAGGGCAGAACAAGAGCC), 5-HT$_{2A}$ (CAGTCCATCAGCAATGAGCAAA, CTGAGCCTGAATATACCGTGAA) and 5-HT$_{2B}$ (CCATCATGCATCTCTGTGCCATTTC, CCATCCAGCATYRCCAYCTTTTC). RT-PCR results were assessed by electrophoresis of the PCR product on ethidium bromide-stained, 1.5% agarose minigel. The expected band was excised. After cDNA extraction from the gel (Gel Extraction Kit; Qiagen, GmbH, Germany), the PCR product was sequenced using standard dideoxy methodologies. The information obtained from the sequencing was compared to other genes from the GenBank using the GCG (Genetics Computer Group) Wisconsin Package (Accelrys, San Diego, CA).

Immunoblot Analysis of Phosphorylated Extracellular Signal-Related Kinase (Erk) 1/2 Activity

Seventy to eighty percent confluent SAVICs were made quiescent by incubation in serum-free M199 overnight. Growth-arrested cells were incubated with or without 10 μmol/L of serotonin for 5 minutes unless otherwise indicated. When selective inhibitors were applied, they were added 30 minutes before the application of serotonin. SAVIC cellular protein was extracted in lysis buffer containing 0.1 mol/L Tris-HCL (pH 8.1), 1% Triton X-114, 10 mmol/L ethylenediaminetetraacetic acid, 0.2 mmol/L sodium orthovanadate, and a mixture of protease inhibitors (Complete Protease Inhibitor Cocktail Tablets; Boehringer Mannheim, GmbH, Mannheim, Germany) and protein concentration was determined by the Bradford method[15] according to the instructions of the manufacturer (BioRad). Ten to 20 μg of total protein from each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis precast gels and then transferred to a polyvinylidene difluoride membrane (BioRad). The membrane was probed with phospho-specific Erk 1/2 antibody (p-p44/42, New England Biolabs) or with a non-phospho-specific Erk 1/2 antibody (p44/42, Santa Cruz Technology).

Statistical Analysis

All values in the text and figures are presented as mean ± SE of three or more independent experiments. Results were analyzed with one-way analysis of variance followed by post test (Tukey-Kramer multiple comparisons test) or Student's *t*-test. A value of $P < 0.01$ was considered statistically significant.

Results

Serotonin Stimulates PLC Activity

PLC activity in cultured SAVICs is induced by serotonin in a dose-dependent (Figure 1A) and time-dependent (Figure 1B) manner. The effects of serotonin on PLC activity were continuously and significantly increased at 1 μmol/L and greater concentrations when serotonin was added to SAVICs for 30 minutes (Figure 1A). PLC activity, while maintaining basal levels (control) at 1 or 5 minutes, was significantly increased after 15 minutes of 10 μmol/L of serotonin exposure. This activity increase was sustained at the longer duration serotonin exposure times (30 and 60 minutes) (Figure 1B). To confirm the specificity of serotonin-stimulated PLC activity, the PLC inhibitor U73122 was used (Figure 1C). U73122 abrogated the serotonin-induced PLC activation. U73122 alone did not change the basal level expression of PLC activity. These data demonstrated the direct association between serotonin and increased PLC activity.

The 5-HT$_{2A}$R Is the Major Serotonin Receptor Subtype in SAVICs

Based on studies with receptor subtype-selective antagonists and agonist, the response of serotonin-stimulated PLC activity appeared to be mediated exclusively by 5-HT$_{2A}$Rs. MDL 100907,[13,16] a selective 5-HT$_{2A}$R antagonist, was able to block completely the serotonin-stimulated PLC activity in a dose-dependent manner (Figure 2A). This decrease was significant ($P < 0.01$) when compared to PLC activity stimulated by serotonin even at the lowest dose (0.1 nmol/L) of MDL 100907 tested. However, SB 242084,[17] a selective antagonist for 5-HT$_{2C}$R (Figure 2B), did not inhibit the serotonin-stimulated PLC activity at any of the concentrations tested. Similarly, SB 206553,[18] a selective antagonist for 5-HT$_{2B/2C}$R (Figure 2C) also had no effect on the serotonin-stimulated PLC activity except at the highest dose tested (1 μmol/L). BW 723C86,[19] a specific 5-HT$_{2B}$R agonist, did not stimulate PLC activity even at 10 μmol/L (Figure 2D). None of the antagonists had any effect on the basal level of PLC activity (control). The data clearly demonstrated that 5-HT$_{2A}$R played an exclusive role in mediating the sero-

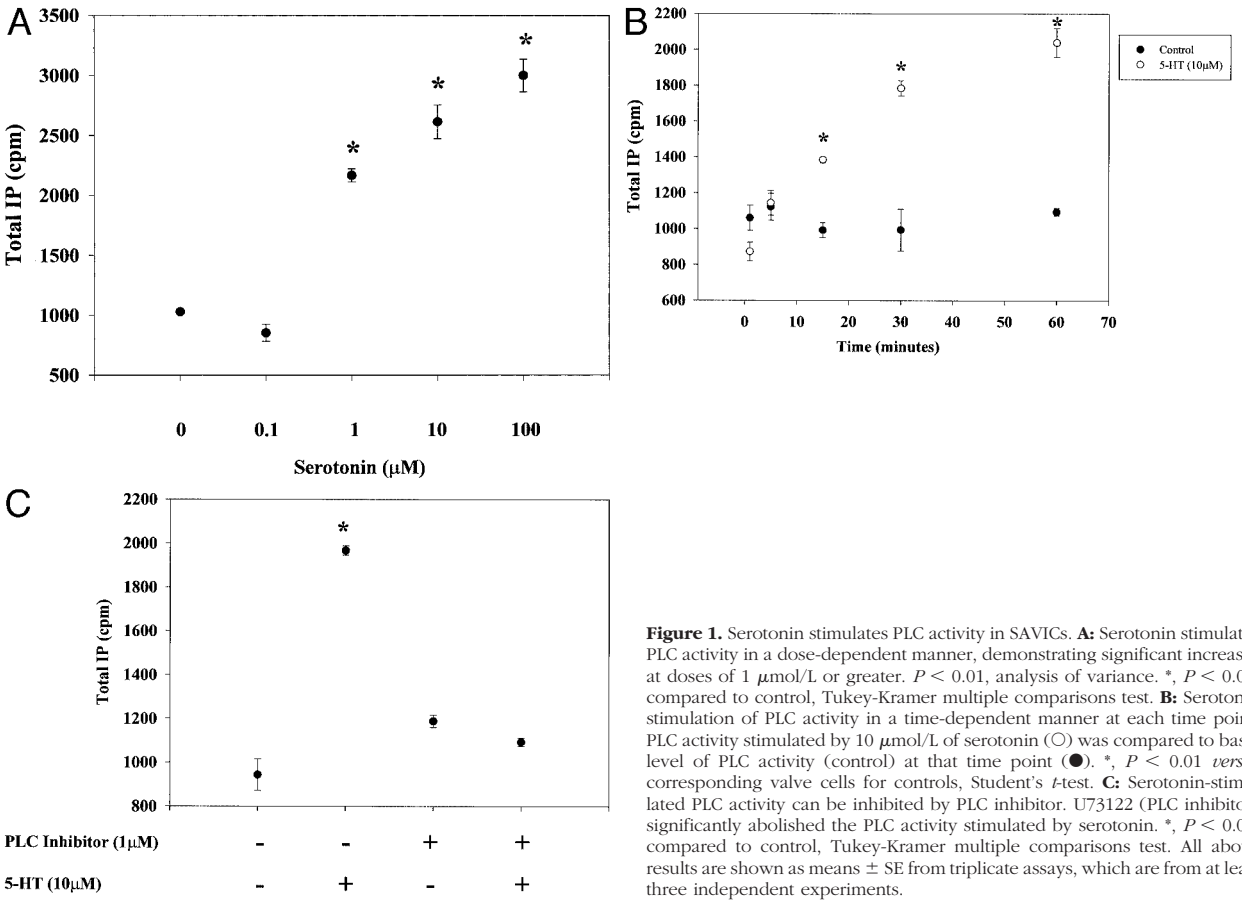

Figure 1. Serotonin stimulates PLC activity in SAVICs. **A:** Serotonin stimulates PLC activity in a dose-dependent manner, demonstrating significant increases at doses of 1 μmol/L or greater. $P < 0.01$, analysis of variance. *, $P < 0.01$, compared to control, Tukey-Kramer multiple comparisons test. **B:** Serotonin stimulation of PLC activity in a time-dependent manner at each time point. PLC activity stimulated by 10 μmol/L of serotonin (○) was compared to basal level of PLC activity (control) at that time point (●). *, $P < 0.01$ *versus* corresponding valve cells for controls, Student's *t*-test. **C:** Serotonin-stimulated PLC activity can be inhibited by PLC inhibitor. U73122 (PLC inhibitor) significantly abolished the PLC activity stimulated by serotonin. *, $P < 0.01$, compared to control, Tukey-Kramer multiple comparisons test. All above results are shown as means ± SE from triplicate assays, which are from at least three independent experiments.

tonin-induced PLC activity whereas 5-$HT_{2B/2C}$R did not appear to be expressed in SAVICs.

We next investigated if 5-HT-induced active TGF-β1 activity could be inhibited by MDL 100907, a selective 5-HT_{2A}R antagonist.[13] In these studies, SAVICs were preincubated with MDL 100907 for 30 minutes, then stimulated by 5-HT for 72 hours. TGF-β1 activity was examined using the PAI/luciferase assay. MDL 100907 significantly inhibited the effects of 5-HT on active TGF-β1 activity in a dose-dependent manner (Figure 3); a significant MDL effect was even observed at the lowest dose (0.1 nmol/L) used ($P < 0.01$). However, MDL 100907 had no significant effect on total TGF-β1 activity (Figure 3B) although an inhibitory trend was noted (Figure 3).

RT-PCR analysis demonstrated that 5-HT_{2A}R was present in normal SAVICs (Figure 4A). Comparison of the partial sheep sequence (Figure 4B) obtained from the cDNA fragment to the 5-HT_{2A}R sequences for porcine and human revealed a highly conserved DNA sequence with 93% and 91% identity, respectively. However, 5-HT_{2B}R was not detectable by PT-PCR in SAVICs using our primers (see above, Materials and Methods) (Figure 4C). Overall, these data have demonstrated that 5-HT_{2A}R mRNA is expressed and is the major functional serotonin receptor subtype in sheep cardiac valvular interstitial cells.

Serotonin Stimulates Erk 1/2 Phosphorylation Activity in SAVICs

Our study also demonstrated that serotonin increased Erk 1/2 phosphorylation activity in AVICs. Serotonin stimulated phosphorylated Erk 1/2 activity in a dose-dependent manner, as detected by specific antibodies against phosphorylated Erk 1/2 in the Western blot analysis (Figure 5A). Compared to the basal level expression of phosphorylated Erk 1/2 activity (control), 100 nmol/L or higher concentrations of serotonin after 5 minutes of exposure to SAVICs caused a notable increase in Erk 1/2 activity. The kinetics of serotonin (10 μmol/L)-induced Erk 1/2 phosphorylation were rapid and transient (Figure 5B). The activated Erk 1/2 reached a peak at 5 minutes and was quickly reduced to basal level activity by 10 minutes. Furthermore, mitogen- and extracellular- signal activated protein kinase kinase (MEK) mediates the Erk 1/2 activation by serotonin, because the MEK inhibitors U0126 and PD98059 completely inhibited the serotonin-stimulated phosphorylation of Erk 1/2 (Figure 6).

However, MDL 100907 (a 5-HT_{2A}R inhibitor) attenuated the 5-HT induced Erk 1/2 activation, but only for Erk-1 and at the highest doses used (Figure 6B). These results suggest that stimulation of Erk 1/2 after 5-HT may be because of activation of other 5-HT receptors and/or

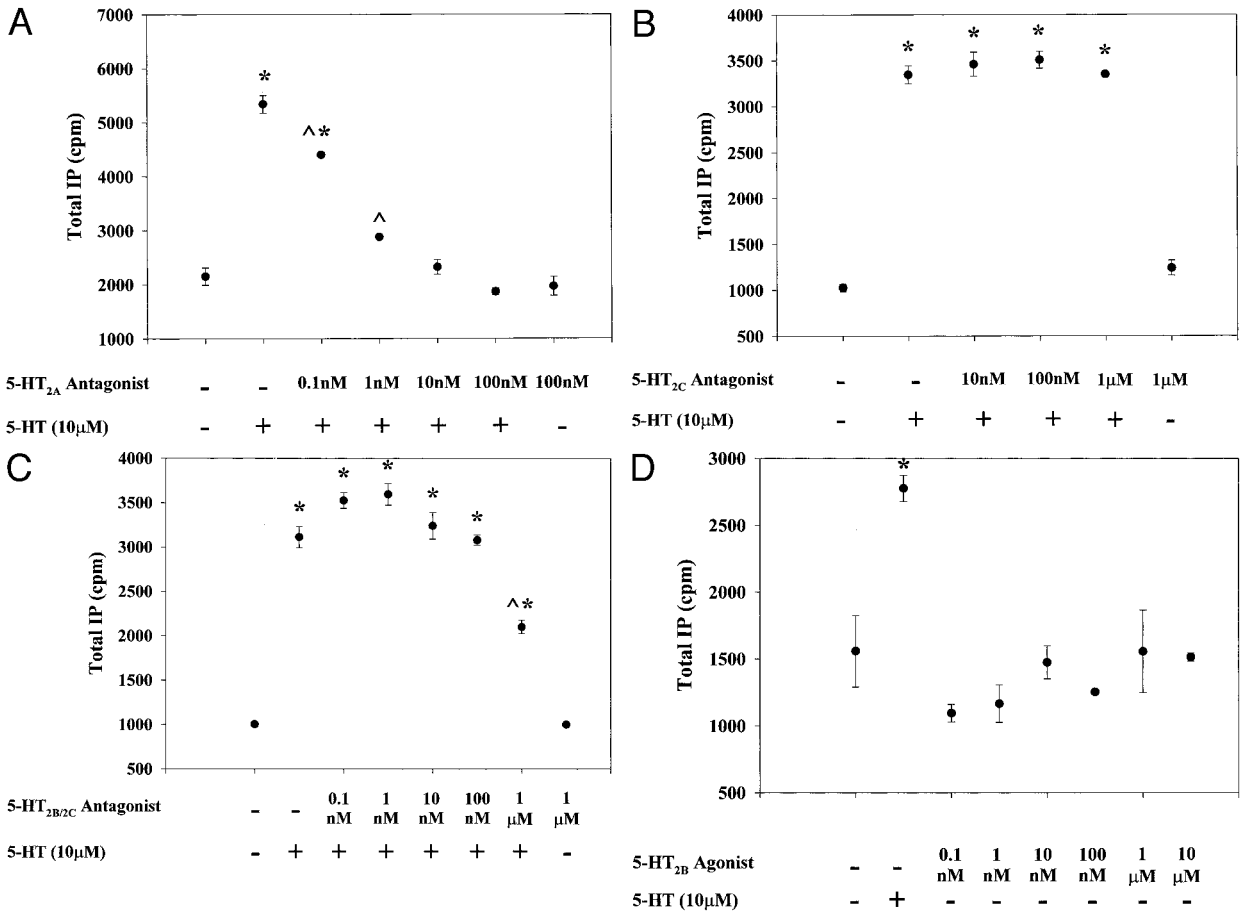

Figure 2. 5-HT$_{2A}$R is the exclusive subtype in SAVICs based on studies with receptor subtype-selective antagonists/agonists, evaluated by PLC assays. **A:** A 5-HT$_{2A}$R-selective antagonist MDL 100907 blocked serotonin-induced PLC activity in a dose-dependent manner. **B:** A 5-HT$_{2C}$R-selective antagonist SB 242084 did not inhibit serotonin-induced PLC activity. **C:** A 5-HT$_{2B/2C}$R-selective antagonist SB 206553 did not inhibit serotonin-induced PLC activity. **D:** A 5-HT$_{2B}$R-selective agonist BW 723W86 did not significantly stimulate PLC activity. Results are shown as means ± SE from triplicates, which are from three independent experiments. $P < 0.01$, analysis of variance from all four figures. *, $P < 0.01$, significant from control; ^, $P < 0.01$, significant from 5-HT treatment alone, Tukey-Kramer multiple comparisons test.

multiple indirect effects rather than solely as a result of 5-HT$_{2A}$R activation.

PKC Is Involved in Serotonin-Erk 1/2 Signaling Pathway

Gα_q is coupled to PLC, and subsequently to protein kinase C (PKC) activation.[20–22] To study the role of PKC in mediating the serotonin-Erk 1/2 signaling pathway in SAVICs, GF109203X, a highly selective PKC inhibitor, was added to SAVICs for half an hour before 5 minutes of 10 μmol/L serotonin exposure. GF109203X attenuated serotonin-stimulated Erk 1/2 activity (Figure 7A), suggesting the involvement of PKC in the signaling pathway from 5-HT$_{2A}$R to Erk 1/2 activation. To further investigate the role of PLC/PKC in this signaling, the effect of overexpression of Gαq on Erk 1/2 phosphorylation activity was determined by using an adenoviral vector construct with constitutively active GTPase-deficient mutant Gα_q (AdCMV-Gα_q). SAVICs were exposed to various concentrations [plaque forming units (PFU)] of AdCMV-Gα_q or AdCMV-GFP (10^8 PFU) overnight. Compared to the effect of AdCMV-GFP on Erk 1/2 activity (similar to the control activity), AdCMV-Gα_q significantly increased Erk 1/2 phosphorylation at a dose of 10^5 PFU. This increase was greater at a higher concentration of AdCMV-Gα_q (10^6 PFU), and reached a plateau thereafter (Figure 7B). The data obtained with AdCMV-Gα_q provided further evidence for the involvement of PKC in mediating Erk 1/2 activation in SAVICs.

Src Tyrosine Kinase Plays a Role in the Serotonin-Erk 1/2 Signaling Pathway

In addition to PKC, Src/Src-like tyrosine kinase has also been demonstrated to be involved in Erk 1/2 activation.[23,24] Various concentrations of pyrazolopyrimidine (PP1, an inhibitor of Src/Src-like tyrosine kinases) were added to SAVICs for 30 minutes before an additional 10 μmol/L of serotonin exposure. This resulted in a dose-dependent decrease in 5-HT-stimulated Erk 1/2 phosphorylation activity (Figure 8). Taken together, our results demonstrated that both PKC and Src/Src-like tyrosine kinase are involved in mediating the stimulatory effects of serotonin on Erk 1/2 activity.

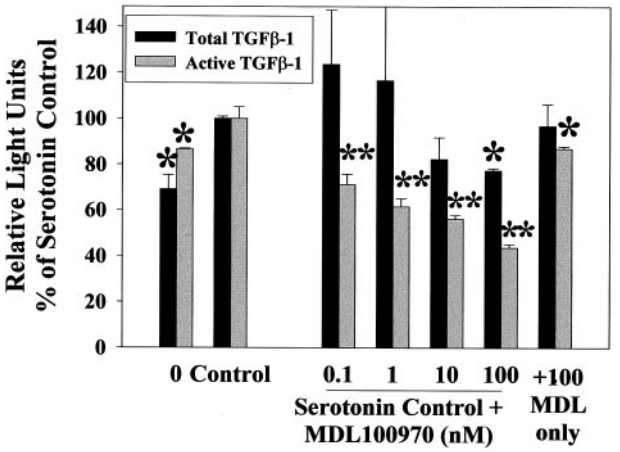

Figure 3. MDL 100970, a 5-HT_{2A}R antagonist, inhibits 5-HT up-regulation of TGF-β activity. 5-HT-induced active TGF-β activity, examined by PAI/luciferase assays, was significantly inhibited by MDL 100970, a selective 5-HT_{2A}R antagonist. (**, $P < 0.01$, significant from 5-HT alone). MDL 100970 had no significant dose-dependent effects on 5-HT-induced total TGF-β activity, examined by PAI/luciferase assays.

Discussion

These studies have demonstrated that 5-HT_{2A}R is the major native functional 5-HT_2R in SAVICs, and that these cells are likely responsive to serotonin via a Gα_q-PLC-PKC signal transduction pathway. Although the exact sequence of signaling events in this pathway leading ultimately to valve disease is unknown, another study by our group has demonstrated that serotonin-induced up-regulation of TGF-β, and a related increase in extracellular matrix components (collagen and glycosaminoglycans), may contribute to the mechanism of serotonin-related heart valve disease.[6] Our studies also showed that the 5-HT_{2A}R antagonist, MDL 100907, inhibited TGF-β1 up-regulation. Thus, the present results strongly support the view that 5-HT-induced heart valve disease may occur via a pathway including the 5-HT_{2A}R with G-protein signal transduction leading to the up-regulation of TGF-β1, as previously demonstrated,[6] with hypothesized associated pathophysiological effects on both cuspal cellular activity and the extracellular matrix.

Seven serotonin receptor subfamilies have been identified to date. The 5-HT_2R subfamily is composed of three members (5-$HT_{2A/2B/2C}$R). Each of them is known to be associated with Gα_q-PLC signal transduction.[24,25] In SAVICs, we have shown the consistent finding of serotonin stimulation of PLC activity in a dose-dependent and time-dependent manner (Figure 1, A and B). The 5-HT_2R-PLC coupling was also demonstrated because a PLC inhibitor U73122 was observed to abolish serotonin-induced PLC activity (Figure 1C).

The expression of the 5-$HT_{2A/2B}$R has been demonstrated by others in human aortic valve cells and porcine aortic valve cells by real-time RT-PCR.[7] Fitzgerald and colleagues[7] demonstrated in human and pig aortic valve cells that there is a predominance of both 5-$HT_{2A/2B}$Rs, with relatively little detectable 5-HT_{2C}R. Furthermore, Roth's group[21] demonstrated a comparable pharmacological response for the 5-HT_{2A} and 5-HT_{2B}Rs for either 5-HT or phenfluoramine. However, it is recognized that right-sided valve involvement predominates in patients

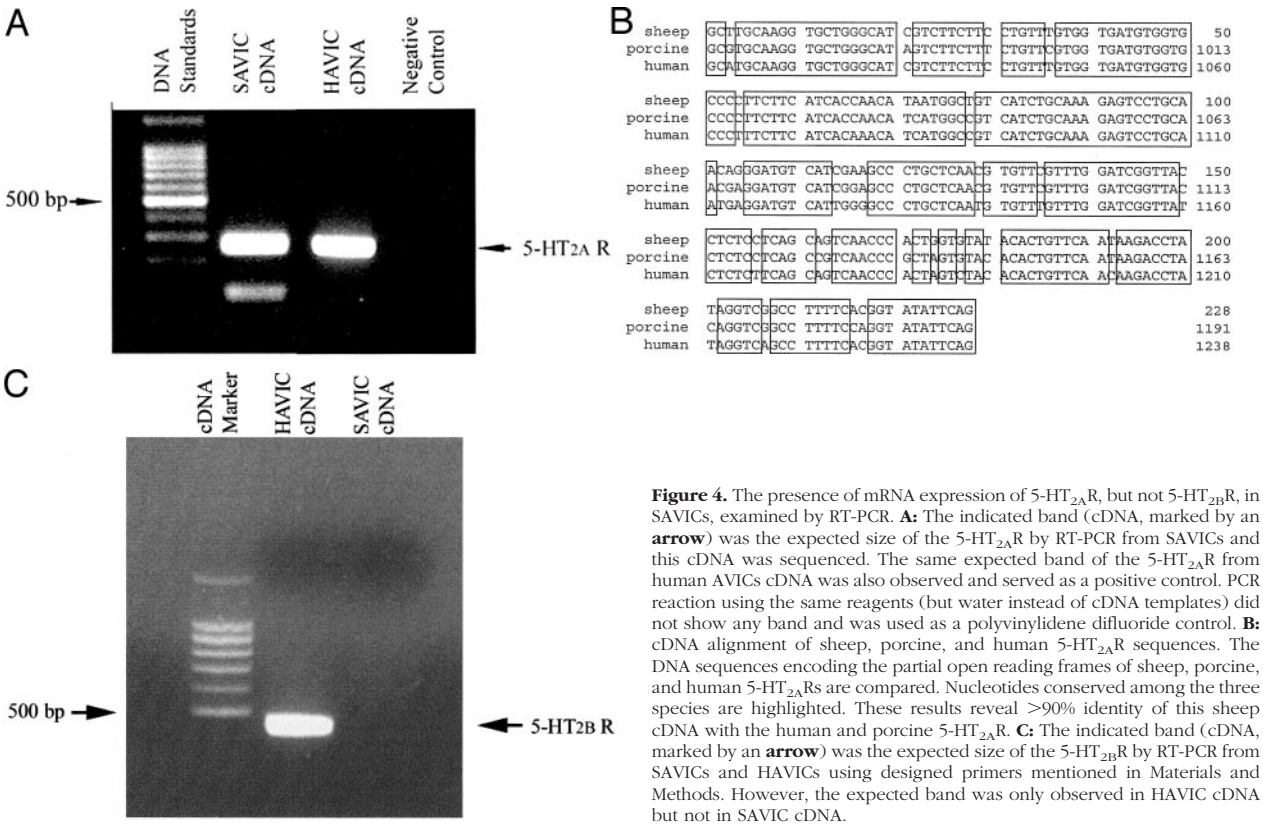

Figure 4. The presence of mRNA expression of 5-HT_{2A}R, but not 5-HT_{2B}R, in SAVICs, examined by RT-PCR. **A:** The indicated band (cDNA, marked by an **arrow**) was the expected size of the 5-HT_{2A}R by RT-PCR from SAVICs and this cDNA was sequenced. The same expected band of the 5-HT_{2A}R from human AVICs cDNA was also observed and served as a positive control. PCR reaction using the same reagents (but water instead of cDNA templates) did not show any band and was used as a polyvinylidene difluoride control. **B:** cDNA alignment of sheep, porcine, and human 5-HT_{2A}R sequences. The DNA sequences encoding the partial open reading frames of sheep, porcine, and human 5-HT_{2A}Rs are compared. Nucleotides conserved among the three species are highlighted. These results reveal >90% identity of this sheep cDNA with the human and porcine 5-HT_{2A}R. **C:** The indicated band (cDNA, marked by an **arrow**) was the expected size of the 5-HT_{2B}R by RT-PCR from SAVICs and HAVICs using designed primers mentioned in Materials and Methods. However, the expected band was only observed in HAVIC cDNA but not in SAVIC cDNA.

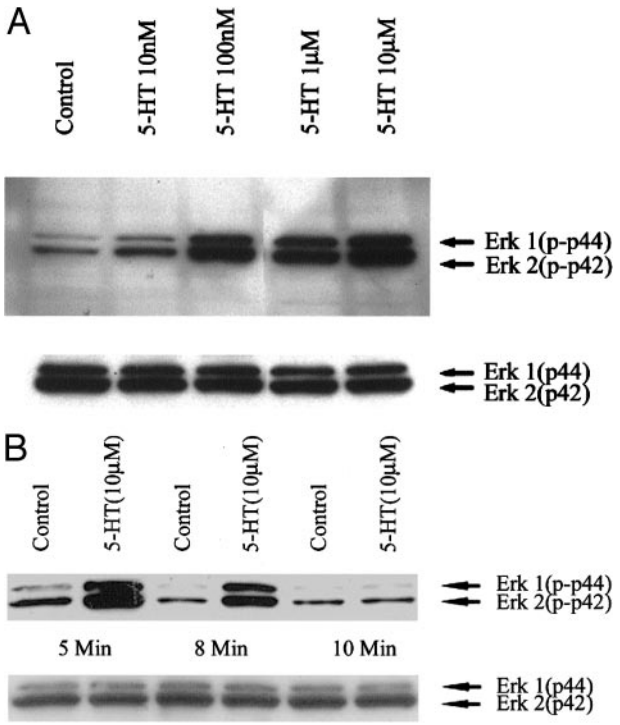

Figure 5. Serotonin stimulates phosphorylation activity of extracellular signal-regulated kinase 1/2 (Erk 1/2), evaluated by Western blot analysis. **A:** Serotonin stimulated Erk 1/2 phosphorylation activity in a dose-dependent manner. **B:** Serotonin stimulated Erk 1/2 phosphorylation activity in a rapid and transient manner. **Top**: Blots were performed using a phospho-specific Erk 1/2 antibody with **arrows** indicating phospho-specific Erk 1 (44 kd, p-p44) and Erk 2 (42 kd, p-p42). **Bottom**: Blots were the stripped **top** blots and reprobed with antibodies detecting nonphosphorylated Erk 1/2, as labeled as p44/p42. They served as loading controls showing the equal amount protein in each lane. **A:** Different concentrations of serotonin are indicated on the **top** of the blot. **B:** Incubation time of SAVICs with 10 μmol/L of serotonin are indicated in the **middle** of the figure, with control and 5-HT labeled on the **top** of the blot in each time point. Data are representative of three or more individual experiments.

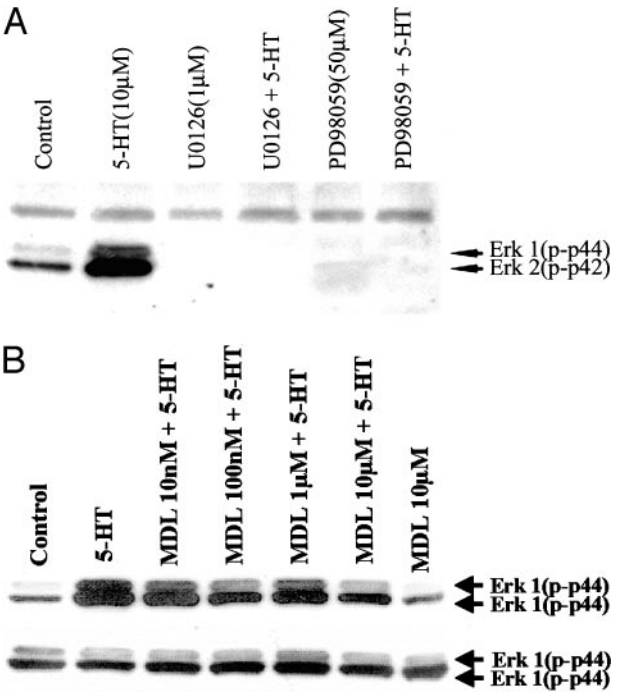

Figure 6. Inhibition of Erk 1/2 phosphorylation activity (per Western blots) by U0126, PD98059, and MDL 100907. **A:** Pretreatment with inhibitors of MEK, U0126, and PD 98059, serotonin (10 μmol/L) stimulated phosphorylation activity of Erk 1/2 (p-p44/p-p42) is totally abolished, indicating the involvement of MEK in serotonin-phospho Erk 1/2 pathway. Various treatments are indicated on the **top** of the blot. **Top bands** are nonspecific bands, serving as loading controls of protein in each lane. This is a representative Western blot of three identical experiments. **B:** The 5-HT$_{2A}$R antagonist (MDL 100907) attenuates the serotonin-induced phosphorylated Erk 1/2 activation. Treatment of the SAVICs with 5-HT$_{2A}$R antagonist, MDL 100907, attenuated the serotonin-stimulated Erk 1/2 phosphorylation activity (p-p44/p-p42). Treatments of the SAVICs with different doses of MDL 100907 are indicated on the **top** of the Western blot. The **bottom** blots were the stripped **top** blots and reprobed with antibodies detecting nonphosphorylated Erk 1/2, as labeled as p44/p42. They served as loading controls. Results from triplicate experiments with comparable results.

with the carcinoid syndrome.[25,26] This is primarily because of the fact that excess serotonin is metabolized to a great extent by pulmonary monoamine oxidase activity, thus sparing left-sided valves from exposure to the highest serotonin levels.[25,26] However, left-sided valve disease has been reported with combined use of serotonergic drugs and monoamine oxidase inhibitors,[25] and left-sided valve disease requiring valve surgery has been reported in patients with carcinoid syndrome.[26] Thus in view of the previous studies concerned with 5-HT$_2$Rs and previous clinical research on left-sided valve disease related to serotonin effects,[25,26] we elected to study AVICs in these initial investigations. Further studies will undertake a complete analysis of pulmonary and tricuspid valve cellular 5-HT responsiveness.

Our studies have used RT-PCR and 5-HT$_2$R subtype-selective antagonists/agonist studies to demonstrate that 5-HT$_{2A}$R was the main functional serotonin receptor present in terms of mRNA/protein levels in SAVICs (Figure 4, A and B, and Figure 2A). Furthermore, 5-HT-induced active TGF-β1 activity was inhibited by MDL 100907, a selective 5-HT$_{2A}$R antagonist (Figure 3A). There is no evidence for the presence of functional 5-HT$_{2B}$ and/or 5-HT$_{2C}$Rs in these cells, because selective antagonists for 5-HT$_{2C}$ and 5-HT$_{2B/2C}$R did not inhibit serotonin-induced PLC activity, and an agonist for 5-HT$_{2B}$R also did not stimulate PLC activity (Figure 2; B, C, and D). Given the fact that only mRNA expression of 5-HT$_{2A/2B}$ was demonstrated (associated with undetectable level of 5-HT$_{2C}$) in human or porcine valve cells from the previous studies,[7] it is possible that activity of 5-HT$_{2B}$R is minimal while 5-HT$_{2B}$R mRNA may still be expressed. In fact, we did not detect 5-HT$_{2B}$R by RT-PCR in SAVICs as well (Figure 4C). However, the presence of 5-HT$_{2B}$R mRNA in SAVICs cannot be ruled out. Because there are no available sheep 5-HT$_{2B}$R sequences in GenBank, the primers designed for checking this receptor subtype contained the consensus sequences from human, porcine, and mouse (positive control of 5-HT$_{2B}$R detected from HAVIC in Figure 4C). Thus, the polyvinylidene difluoride results of our RT-PCR studies may be because of relatively large variations in sequences among the species for 5-HT$_{2B}$R subtypes.

Serotonin has been shown to increase the production of collagen in mesangial cells through the actions of protein kinase C (PKC) and TGF-β1 via the G-protein-PLC signaling pathway.[27,28] 5-HT$_2$R signal transduction has also been reported to regulate extracellular signal-

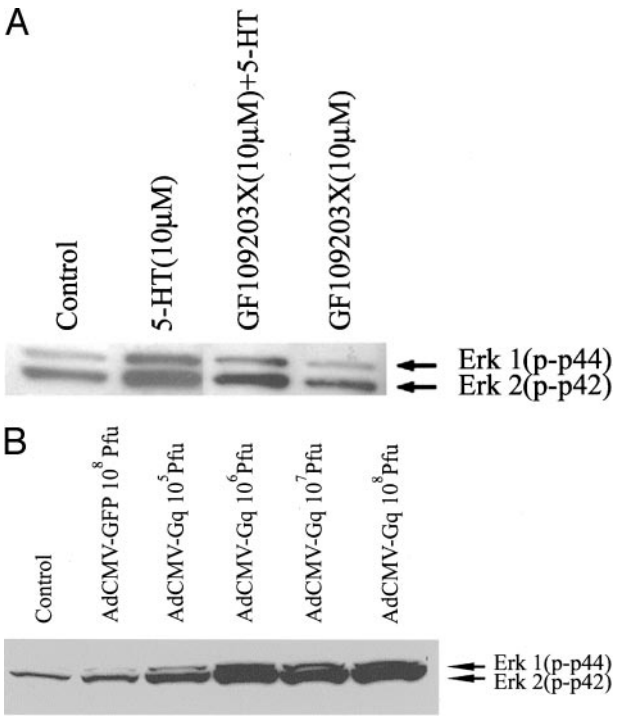

Figure 7. Protein kinase C (PKC) is one of the components involving the serotonin-phospho Erk 1/2 pathway in SAVICs, evaluated by Western blot analysis. **A:** GF109203X, a PKC inhibitor, reduces serotonin-induced Erk 1/2 phosphorylation activity. Effect of GF109203X on the serotonin-induced Erk 1/2 phosphorylation (p-p44/p-p42) in SAVICs was determined. Treatments are indicated at the **top** of the blot. This is a representative Western blot of three identical experiments. **B:** Overexpression of activated Gαq via transduction of its cDNA with a replication-defective adenovirus results in increasing of Erk 1/2 phosphorylation. Adenoviral constitutively active GTPase-deficient mutant Gαq (AdCMV-Gαq) stimulates Erk 1/2 phosphorylation activity (p-p44/p-p42) in a dose-dependent manner. AdCMV-Gq 10^5 PFU to AdCMV-Gq 10^8 PFU are indicated at the **top** of the blot. Adenoviral GFP (AdCMV-GFP 10^8 PFU) was used as a polyvinylidene difluoride control. This is a representative Western blot of two identical experiments.

regulated kinase 1/2 (Erk 1/2) activation in smooth muscle cells, fibroblasts, and mesangial cells.[27,29,30] Erk 1/2, a mitogen-activated protein kinase (MAPK), is involved in signal transduction from the cell surface to the nucleus. Phosphorylated active Erk 1/2 has been demonstrated to increase the production of extracellular matrix components such as collagen in many cell types including cardiomyocytes and fibroblasts.[31–35] Thus, our study investigated whether Erk 1/2 can be activated by serotonin

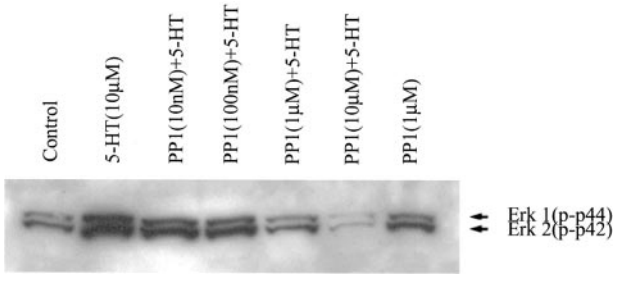

Figure 8. Src kinase inhibitor PP1 blocked serotonin-induced Erk 1/2 phosphorylation activity in SAVICs. The blocking effect of Src kinase inhibitor PP1 on serotonin-induced Erk 1/2 (p-p44/p-p42) was observed to be dose-dependent. Different treatments are indicated on the **top** of the blot. Shown are representative results from three identical experiments.

in SAVIC cultures. The present studies demonstrated a strong association of serotonin and Erk 1/2 phosphorylation in AVICs (Figure 5) using concentrations of 5-HT in the same range demonstrated to stimulate a TGF-β1-dependent increase in proline incorporation.[16] The serotonin-induced Erk 1/2 activation was dose-dependent (Figure 5A) and transient (Figure 5B). These rapid and transient signaling results are comparable to those observed in other cell types.[36,37] However, 5-HT-induced TGF-β1 RNA synthesis in SAVICs occurs with 3 hours of 5-HT addition (Figure 3)[6] and thus, could reflect the cumulative effects of 5-HT > Erk 1/2 signaling. It was also observed that increased serotonin-induced Erk 1/2 phosphorylation was only minimally decreased by the 5-HT_{2A}R-specific antagonist MDL 100907 (Figure 6B) at the relatively high concentrations of 100 nmol/L or more. A limited effect was seen mostly on Erk1 phosphorylation.

Thus, it is likely that other 5-HT receptors are present that are responsible for serotonin-induced Erk 1/2 phosphorylation. Although Erk 1/2 is well known for involvement in mitogenic signaling,[38,39] 5-HT had no effects on proliferation in the present studies. Nevertheless, there have been investigations by others[40,41] demonstrating the involvement of Erk 1/2 in signaling pathways that did not involve mitogenic activity.[40,41] Thus, 5-HT could activate multiple pathways in SAVICs, and those involving either Erk 1/2 or TGF-β could be unrelated.

PKC was shown to be involved in mediating the serotonin-induced Erk 1/2 activation based on two lines of evidence: GF109203X, a PKC inhibitor, reduced Erk 1/2 activity induced by serotonin (Figure 7A); and constitutively active, GTPase-deficient mutant $G\alpha_q$ adenoviral vector[42] stimulated Erk 1/2 activity in SAVICs (Figure 7B). In addition to PKC, Src or Src-like tyrosine kinase also appeared to play an important role in the serotonin-mediated Erk 1/2 activation because the Src inhibitor PP1[43] reduced 5-HT-stimulated Erk 1/2 activity in a dose-dependent manner (Figure 7). Whether this effect is related to the association of PKC/Src complex[44] or to a PKC-independent signaling pathway involving Src[45] in SAVICs remains to be resolved.

The present data have a number of important implications. Because the abnormalities in the serotonin/serotonergic system may play an important role in heart valve disease, 5-HT_2 serotonergic receptor blockers, such as the selective 5-HT_{2A}R antagonist, MDL 100907,[13,16] may eventually have therapeutic potential in the treatment of heart valve diseases. Furthermore, numerous pharmacologically relevant drugs could be screened in AVIC culture systems to determine the existence of potential stimulatory effects on the 5-HT_{2A}R-$G\alpha_q$-PLC pathway.

Conclusion

Our studies have functionally characterized the signal transduction activity of AVICs in response to 5-HT revealing that the 5-HT_{2A}Rs are the most active of the 5-HT_2Rs in this cell type. Furthermore, the 5-HT_{2A}R also appears to be responsible for 5-HT-mediated increased TGF-β activity in SAVICs that may contribute to progression of

5-HT-related heart valve disease. However, 5-HT also mediated strong Erk 1/2 signaling via the MAP-kinase pathway that was only in part because of 5-HT$_{2A}$R activity. These results indicate major 5-HT Erk 1/2 signaling beyond that mediated by 5-HT$_2$Rs that must involve other serotonin receptor types and/or secondary signaling events.

Acknowledgments

We thank Ms. Xiumin Cui, Ms. Michelle Lee, and Mrs. Jeanne Connolly for their technical assistance; Ms. Jennifer LeBold for her assistance in the preparation of this manuscript; Dr. Morris Birnbaum (University of Pennsylvania) for providing AdCMV-Gα_q; and Dr. D. B. Rifkin (New York University) for providing his mink lung epithelial cells for TGF-β assays.

References

1. Waltenberger J, Lundin L, Oberg K, Wilander E, Miyazono K, Heldin CH, Funa K: Involvement of transforming growth factor-beta in the formation of fibrotic lesions in carcinoid heart disease. Am J Pathol 1993, 142:71–78
2. Robiolio PA, Rigolin VH, Wilson JS, Harrison JK, Sanders LL, Bashore TM, Feldman JM: Carcinoid heart disease. Correlation of high serotonin levels with valvular abnormalities detected by cardiac catheterization and echocardiography. Circulation 1995, 92:790–795
3. Kulke MH, Mayer RJ: Carcinoid tumors. N Engl J Med 1999, 340:858–868
4. Ferrans VJ, Roberts WC: The carcinoid endocardial plaque; an ultrastructural study. Hum Pathol 1976, 7:387–409
5. Hafizi S, Taylor PM, Chester AH, Allen SP, Yacoub MH: Mitogenic and secretory responses of human valve interstitial cells to vasoactive agents. J Heart Valve Dis 2000, 9:454–458
6. Jian B, Xu J, Savani R, Narula N, Liang B, Levy R: Serotonin mechanisms in heart valve disease I. Serotonin induced upregulation of TGF-B1 via G-protein signal transduction in aortic valve interstitial cells. Am J Pathol 2002, 161:2207–2216
7. Fitzgerald LW, Burn TC, Brown BS, Patterson JP, Corjay MH, Valentine PA, Sun JH, Link JR, Abbaszade I, Hollis JM, Largent BL, Hartig PR, Hollis GF, Meunier PC, Robichaud AJ, Robertson DW: Possible role of valvular serotonin 5-HT(2B) receptors in the cardiopathy associated with fenfluramine. Mol Pharmacol 2000, 57:75–81
8. Roy A, Brand NJ, Yacoub MH: Expression of 5-hydroxytryptamine receptor subtype messenger RNA in interstitial cells from human heart valves. J Heart Valve Dis 2000, 9:256–261
9. Greene EL, Houghton O, Collinsworth G, Garnovskaya MN, Nagai T, Sajjad T, Bheemanathini V, Grewal JS, Paul RV, Raymond JR: 5-HT (2A) receptors stimulate mitogen-activated protein kinase via H(2)O(2) generation in rat renal mesangial cells. Am J Physiol 2000, 278:F650–F658
10. Nebigil CG, Launay JM, Hickel P, Tournois C, Maroteaux L: 5-hydroxytryptamine 2B receptor regulates cell-cycle progression: cross-talk with tyrosine kinase pathways. Proc Natl Acad Sci USA 2000, 97:2591–2596
11. Rosel P, Arranz B, San L, Vallejo J, Crespo JM, Urretavizcaya M, Navarro MA: Altered 5-HT(2A) binding sites and second messenger inositol trisphosphate (IP(3)) levels in hippocampus but not in frontal cortex from depressed suicide victims. Psychiatry Res 2000, 99:173–181
12. Berridge MJ, Dawson RM, Downes CP, Heslop JP, Irvine RF: Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem J 1983, 212: 473–482
13. Svartengren J, Simonsson P: Receptor binding properties of amperozide. Pharmacol Toxicol 1990, 66(Suppl)1:S8–S11
14. Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB: An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. Anal Biochem 1994, 216:276–284
15. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976, 72:248–254
16. Baxter G, Kennett G, Blaney F, Blackburn T: 5-HT2 receptor subtypes: a family reunited? Trends Pharmacol Sci 1995, 16:105–110
17. Kennett GA, Wood MD, Bright F, Trail B, Riley G, Holland V, Avenell KY, Stean T, Upton N, Bromidge S, Forbes IT, Brown AM, Middlemiss DN, Blackburn TP: SB 242084, a selective and brain penetrant 5-HT2C receptor antagonist. Neuropharmacology 1997, 36:609–620
18. Kennett GA, Wood MD, Bright F, Cilia J, Piper DC, Gager T, Thomas D, Baxter GS, Forbes IT, Ham P, Blackburn TP: In vitro and in vivo profile of SB 206553, a potent 5-HT2C/5-HT2B receptor antagonist with anxiolytic-like properties. Br J Pharmacol 1996, 117:427–434
19. Kennett GA, Ainsworth K, Trail B, Blackburn TP: BW 723C86, a 5-HT2B receptor agonist, causes hyperphagia and reduced grooming in rats. Neuropharmacology 1997, 36:233–239
20. Taylor SJ, Chae HZ, Rhee SG, Exton JH: Activation of the beta 1 isozyme of phospholipase C by alpha subunits of the Gq class of G proteins. Nature 1991, 350:516–518
21. Roth BL, Willins DL, Kristiansen K, Kroeze WK: 5-Hydroxytryptamine2-family receptors (5-hydroxytryptamine2A, 5-hydroxytryptamine2B, 5-hydroxytryptamine2C): where structure meets function. Pharmacol Ther 1998, 79:231–257
22. Jalili T, Takeishi Y, Walsh RA: Signal transduction during cardiac hypertrophy: the role of G alpha q, PLC beta I, and PKC. Cardiovasc Res 1999, 44:5–9
23. Lopez-Ilasaca M: Signaling from G-protein-coupled receptors to mitogen-activated protein (MAP)-kinase cascades. Biochem Pharmacol 1998, 56:269–277
24. Luttrell LM, Hawes BE, van Biesen T, Luttrell DK, Lansing TJ, Lefkowitz RJ: Role of c-Src tyrosine kinase in G protein-coupled receptor- and Gbetagamma subunit-mediated activation of mitogen-activated protein kinases. J Biol Chem 1996, 271:19443–19450
25. Connolly HM, Crary JL, McGoon MD, Hensrud DD, Edwards BS, Edwards WD, Schaff HV: Valvular heart disease associated with fenfluramine-phentermine. N Engl J Med 1997, 337:581–588
26. Connolly HM, Schaff HV, Mullany CJ, Rubin J, Abel MD, Pellikka PA: Surgical management of left-sided carcinoid heart disease. Circulation 2001, 104:I36–I40
27. Grewal JS, Mukhin YV, Garnovskaya MN, Raymond JR, Greene EL: Serotonin 5-HT2A receptor induces TGF-beta1 expression in mesangial cells via ERK: proliferative and fibrotic signals. Am J Physiol 1999, 276:F922–F930
28. Kasho M, Sakai M, Sasahara T, Anami Y, Matsumura T, Takemura T, Matsuda H, Kobori S, Shichiri M: Serotonin enhances the production of type IV collagen by human mesangial cells. Kidney Int 1998, 54:1083–1092
29. Florian JA, Watts SW: Integration of mitogen-activated protein kinase kinase activation in vascular 5-hydroxytryptamine2A receptor signal transduction. J Pharmacol Exp Ther 1998, 284:346–355
30. Hahn A, Heusinger-Ribeiro J, Lanz T, Zenkel S, Goppelt-Struebe M: Induction of connective tissue growth factor by activation of heptahelical receptors. Modulation by Rho proteins and the actin cytoskeleton. J Biol Chem 2000, 275:37429–37435
31. Su B, Karin M: Mitogen-activated protein kinase cascades and regulation of gene expression. Curr Opin Immunol 1996, 8:402–411
32. Sugden PH, Clerk A: Regulation of the ERK subgroup of MAP kinase cascades through G protein-coupled receptors. Cell Signal 1997, 9:337–351
33. Treisman R: Regulation of transcription by MAP kinase cascades. Curr Opin Cell Biol 1996, 8:205–215
34. Yue TL, Gu JL, Wang C, Reith AD, Lee JC, Mirabile RC, Kreutz R, Wang Y, Maleeff B, Parsons AA, Ohlstein EH: Extracellular signal-regulated kinase plays an essential role in hypertrophic agonists, endothelin-1 and phenylephrine-induced cardiomyocyte hypertrophy. J Biol Chem 2000, 275:37895–37901
35. Fringer J, Grinnell F: Fibroblast quiescence in floating or released collagen matrices: contribution of the ERK signaling pathway and actin cytoskeletal organization. J Biol Chem 2001, 276:31047–31052
36. Goppelt-Struebe M, Hahn A, Stroebel M, Reiser CO: Independent

regulation of cyclo-oxygenase 2 expression by p42/44 mitogen-activated protein kinases and Ca2+/calmodulin-dependent kinase. Biochem J 1999, 339:329–334
37. Chandler LJ, Sutton G, Dorairaj NR, Norwood D: N-methyl D-aspartate receptor-mediated bidirectional control of extracellular signal-regulated kinase activity in cortical neuronal cultures. J Biol Chem 2001, 276:2627–2636
38. Lee SL, Simon AR, Wang WW, Fanburg BL: H2O2 signals 5-HT induced ERK MAP kinase activation and mitogenesis of smooth muscle cells. Am J Physiol 2001, 281:L646–L652
39. Locher R, Brandes RP, Vetter W, Barton M: Native LDL induces proliferation of human vascular smooth muscle cells via redox-mediated activation of Erk 1/2 mitogen-activated protein kinases. Hypertension 2002, 39:645–650
40. Jiang F, Jia Y, Cohen I: Fibronectin and protein kinases C-mediated activation of ERK/MAPK are essential for proplatelike formation. Blood 2002, 99:3579–3584
41. Gu J, Fujibayashi A, Yamada KM, Sekiguchi K: Laminin-10/11 and fibronectin differentially prevent apoptosis by serum removal via phosphatidylinositol 3-kinase/Akt- and MEK1/ERK-dependent pathways. J Biol Chem 2002, 277:19922–19928
42. Cummins MM, Poronnik P, O'Mullane LM, Cook DI: Studying heterotrimeric G-protein-linked signal transduction using replication-deficient adenoviruses. Immunol Cell Biol 2000, 78:375–386
43. Vondriska TM, Zhang J, Song C, Tang XL, Cao X, Baines CP, Pass JM, Wang S, Bolli R, Ping P: Protein kinase C epsilon-Src modules direct signal transduction in nitric oxide-induced cardioprotection: complex formation as a means for cardioprotective signaling. Circ Res 2001, 88:1306–1313
44. Akhand AA, Pu M, Senga T, Kato M, Suzuki H, Miyata T, Hamaguchi M, Nakashima I: Nitric oxide controls src kinase activity through a sulfhydryl group modification-mediated Tyr-527-independent and Tyr-416-linked mechanism. J Biol Chem 1999, 274:25821–25826
45. Gerhardt CC, van Heerikhuizen H: Functional characteristics of heterologously expressed 5-HT receptors. Eur J Pharmacol 1997, 334: 1–23